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The effects of chronic bile reflux on the gastric mucosa of rats

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Background/aims: *To establish a rat model mimicking human bile reflux for studying the pathological effects of chronic bile reflux.* ***Materials and Methods:*** *The duodenum of Sprague–Dawley rats was transected below the opening of the common bile duct, and a gastrojejunostomy was performed at the greater curvature of the forestomach. After the rats demonstrated bile reflux for 1 year, we studied the pathological features of the glandular stomach and forestomach mucosa. We also studied the effect of bile reflux on gastrin expression in the glandular stomach mucosa by using immunohistochemistry.* ***Results:*** *Chronic bile reflux caused significant hyperplasia and expansion of gastric glands in the glandular stomach. Dysplasia and cancer formation also developed, but the incidence was significantly lower than that reported in the literature. Intestinal metaplasia and ulceration in the glandular stomach were also rare. In the forestomach, the squamous epithelium showed significant hyperplasia and keratinization along with keratin pearls and keratocysts. Intestinal metaplasia was rare and no tumorigenesis was observed. Chronic bile reflux significantly increased gastrin expression in the glandular stomach mucosa.* ***Conclusions:*** *When simulating the physiological bile reflux pathway, chronic bile reflux caused hyperplasia and expansion of gastric glands in the glandular stomach and squamous epithelial hyperplasia and keratinization in the forestomach.*

Key words: Bile reflux, bile, adenocarcinoma, intestinal metaplasia, gastrin

Sıçanlarda kronik safra reflüsünün gastrik mukoza üzerine olan etkileri

Amaç: *Kronik safra reflüsünün etkilerini araştırmak amacı ile hayvan modeli oluşturması amaçlanmıştır.* ***Gereç ve Yöntem:*** *Sprague-Dawley cinsi sıçanların duodenumu safra duktusunun açılımının distalinden diseke edildi. midenin büyük kurvaturuna gastrojejunostomi anastomozu gerçekleştirildi. Bir yıl kadar safra reflüsü ile izlendikten sonra mide mukozasındaki patolojik değişiklikleri incelemek üzere glandüler ve ön mide mukozaları incelendi. Ayrıca, safra reflüsünün glandüler mukozadaki gastrin ekspresyonuna olan etkisini incelemek için immunohistokimyasal yöntemler kullanıldı.* ***Bulgular:*** *Kronik safra reflüsü, glandüler gastrik mukoza üzerinde belirgin hiperplatik ve ekspansiyona neden olmaktaydı. Displazi ve kanser gelişimi de gözlendi ancak insidansı literatürdekine göre düşük bulundu. İntestinal metaplazi ve ülser gelişimi de nadir olarak gözlendi. Ön midede skuamöz epitelde anlamlı hiperplazi, keratinizasyon ile beraber keratin incileri ve keratokistler izlendi. Ayrıca intestinal metaplazi nadirdi ve tümörogenez gözlenmedi. Kronik safra reflüsünün glandüler mukozada gastrin ekspresyonunda anlamlı artışa neden olduğu görüldü.* ***Sonuç:*** *Fizyolojik safra reflüsü taklit edildiği yöntemde, kronik safra reflüsü glandüler mide mukozasında hiperplaziye ve ekspansiyona neden olurken, skuamöz epitelde hiperplazi ve keratinizasyona neden olmaktadır.*

Anahtar kelimeler: Safra reflüsü, safra, adenokarsinoma, intestinal metaplazi, gastrin

INTRODUCTION

There is a longstanding clinical observation that subjects who have undergone gastrectomy or other surgeries associated with free reflux of bile into the stomach or esophagus have increased suscep-

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tibility to gastric or esophageal cancers. Other experimental models of bile reflux have shown that bile reflux produces gastric or esophageal cancers. However, the effect of chronic bile reflux on the stomach and esophageal mucosa of humans without foregut surgery is unclear. The clinical application of Bilitec 2000 technology for the 24 h bile reflux monitoring revealed that bile reflux into the stomach or even the esophagus was a frequent phenomenon (1-3). Dixon et al. believed that bile reflux might be related to the intestinal metaplasia of gastric antral mucosa (4-6). Recent studies have shown that bile reflux is not only related to reflux esophagitis but also is a significant cause of Barrett's esophagus (7-11) and esophageal adenocarcinoma (12-17). Therefore, studying the pathological effects of chronic bile reflux on gastric and esophageal mucosa in subjects without foregut surgery has clinical significance. In this study, we established a rat model that simulated the physiological bile reflux pathway shown by humans, so as to mimic bile reflux in humans without foregut surgery, in order to study the pathological effects of chronic bile reflux on the gastric mucosa of rats. We also studied the effect of chronic bile reflux on gastrin expression by using immunohistochemistry, and probed the role of gastrin expression in bile reflux.

MATERIALS and METHODS

Animals and antibodies

Eight-week-old, male Sprague–Dawley rats (clean grade) weighing 250-300 g were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences. Goat anti-rat gastrin polyclonal IgG (Santa Cruz Company, USA) and rabbit anti-goat IgG (Beijing Zhongshan Company, China) were used. The protocols in this study were approved by the Institutional Review Board and the Animal Care and Use Committee of Affiliated Putuo Hospital of Shanghai University of Traditional Chinese Medicine (Shanghai, China).

Animal model

The rat stomach is composed of two parts: the proximal forestomach and the distal glandular stomach. The forestomach mucosa, like the human esophagus, is covered with squamous epithelium. The glandular stomach is equivalent to that of humans, and can be divided into the gastric body and antrum. The rats were acclimatized for 1 week and fasted for 24 h before surgery. Thereafter, only 5% glucose water was provided until 6 h before surgery. Chloral hydrate (0.3 ml/100 g; 10%) was injected intraperitoneally to anesthetize the rats. A 4 cm incision was made at the epigastric midline. The duodenum was transected 0.5 cm below the opening of the common bile duct, and the ends sutured. One cm incisions were made 2 mm proximal to the junction of the forestomach and the glandular stomach, at the greater curvature of the forestomach, and at 4 cm distal to the ligament of Treitz at the contralateral side of the jejunal mesentery. Thereafter, gastrojejunostomy was performed. The rats were fasted for 24 h after the operation; only 5% glucose water was provided. A small amount of feed was provided after 24 h, and normal diet was provided after 3 days. A group of ten rats without surgery housed with the experimental rats for 1 year served as controls.

Histopathological examination

A year after the operation, a total of 13 model rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.3 ml/100 g) and sacrificed by decapitation. Rat stomachs were fixed in 10% formalin for 72 h, embedded in paraffin, sliced (thickness, 4 μm), and stained (hematoxylin and eosin [H&E] staining).

Immunohistochemistry

The sections were incubated at 65°C for 1 h, then dewaxed, hydrated, washed with running water for 10 min, washed with distilled water twice, and immersed in distilled water for 5 min. Then, the sections were incubated in 3% H_2O_2 for 10 min at room temperature to eliminate endogenous peroxidase activity, washed with running water for 10 min, washed with distilled water twice, and immersed in distilled water for 5 min. The sections were then treated with microwaves for 15 min for epitope retrieval in 0.01 M citric acid buffer, washed with phosphate-buffered saline (PBS) (5 min, 3 times), and incubated in serum for 30 min at room temperature to block the binding sites of the endogenous antigen. The sections were incubated with the primary antibodies diluted in PBS (1:100) overnight at 4°C. Then, the sections were washed with PBS (5 min, 3 times) and incubated with the secondary antibodies for 1 h at room temperature. Then, the sections were washed with PBS (5 min, 3 times) and incubated with horseradish peroxidase for 1 h at room temperature. After 1 h, the following steps were performed: the sections were

washed with PBS (5 min, 3 times), incubated with diaminobenzidine (DAB) developer for 6 sec, washed with running water for 10 min, counterstained with hematoxylin for 20 sec, washed with running water for 5 min, dipped in hydrochloric acid for 1 sec, washed with running water for 5 min, dried at room temperature, and mounted using neutral gum.

RESULTS

Histopathology

Only mild chronic inflammation of the stomach was found in a few rats after 1 year in the control group.

However, after all 13 model rats showed bile reflux for 1 year, significant hyperplasia of the glands of the glandular stomach was observed; most rats also showed significant expansion of the glands (Table 1, Figure 1 A-D). Chronic bile reflux caused glandular dysplasia in 3 rats, 1 of which had cancer. Erosion and ulceration were relatively rare and observed in 3 rats and 1 rat, respectively. Similarly, intestinal metaplasia was rare and observed only in 1 rat.

After the rats showed bile reflux for 1 year, significant hyperplasia and keratinization of the forestomach squamous epithelium was observed in all 13 rats. Seven rats had large keratin pearls at the submucosa, and 3 rats had keratocysts (Table 1, Figure 2 A-D). Similar to the metaplasia in the glandular stomach, intestinal metaplasia was relatively rare and observed only in 1 rat. Erosion and ulceration were not observed in the forestomachs of any rats.

Immunohistochemistry

In the control rats, gastrin-positive cells were mainly observed in the glandular gastric antral epithelium, and the number of cells was very small. After the rats showed bile reflux for 1 year, significant hyperplasia and aggregation were observed in the gastrin-positive cells of the glandular gastric antral epithelium (Figure 3 A-F). Hyperplasia was also observed in the gastrin-positive cells of the epithelium of the glandular stomach body.

DISCUSSION

As mentioned above, the rat stomach is composed of the proximal forestomach and the distal glandular stomach. The forestomach mucosa, like the human esophagus, is covered with squamous epithelium. The glandular stomach is like that of humans, and can be divided into the gastric body and antrum. Our rat model is a revised version of that used by Kaminishi et al. (18). In our model rats, the bile flowed through the pylorus into the glandular stomach and then into the jejunum through the gastrojejunal stoma at the forestomach. The entire glandular stomach was kept intact, therefore, the bile reflux pathway in our rats was similar to that in humans without foregut surgery, and the effect of chronic bile reflux on both the glandular epithelium and squamous epithelium could be studied simultaneously.

In this study, we found that chronic bile reflux caused hyperplasia of the foveolar epithelium of the body and antrum of rat glandular stomachs; this finding is consistent with the description of reactive gastritis suggested at the Houston Conference in 1994 (19). Furthermore, all the rats sho-

Table 1. Pathological features of the rats who showed chronic bile reflux

Pathological features	Number (n = 13)
Glandular stomach	
Hyperplasia and expansion of the gland	13
Dysplasia	3
Canceration	1
Erosion	3
Ulcer	1
Intestinal metaplasia	1
Forestomach	
Hyperplasia and keratinization of the forestomach squamous epithelium	13
Keratin pearl	7
Keratocyst	3
Intestinal metaplasia	1

wed hyperplasia and cystic dilatation of the glands of the glandular stomach; this finding is consistent with those of Taylor et al. (20) and Mukaisho et al. (21), suggesting that hyperplasia and expansion of the gastric glands is also a characteristic feature of gastritis induced by bile reflux.

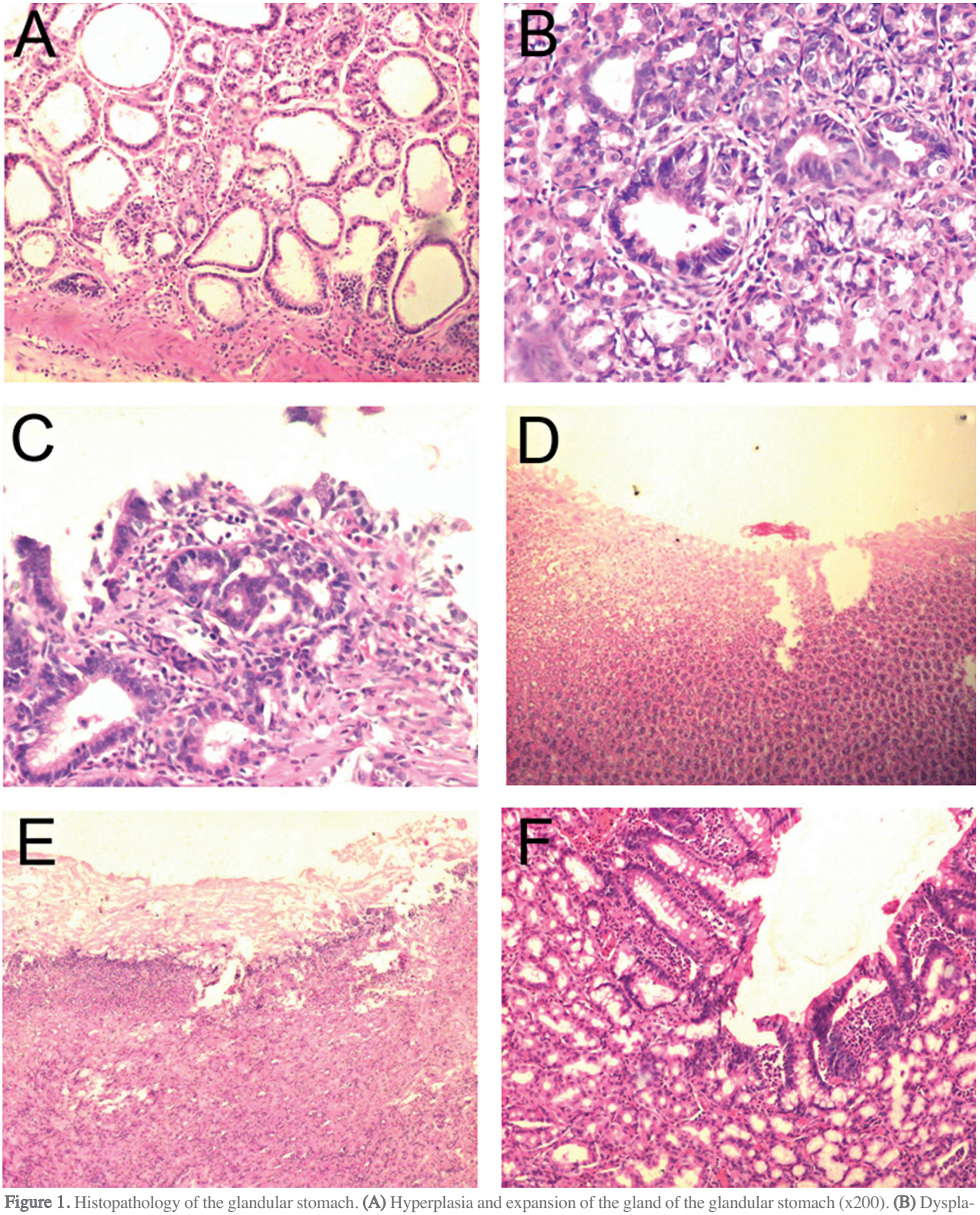

Figure 1. Histopathology of the glandular stomach. **(A)** Hyperplasia and expansion of the gland of the glandular stomach (x200). **(B)** Dysplasia of the gland of the glandular stomach (x400). **(C)** Canceration of the gland of the glandular stomach (x400). **(D)** Erosion of the glandular stomach mucosa (x200). **(E)** Ulcer in the glandular stomach (x200). **(F)** Intestinal metaplasia of the gland of the glandular stomach (x200).

In our model, only 1 case of glandular-stomach cancer formation was found, which is much lower than observed in other studies (20-23). This difference may be attributable to the different operations performed in the studies. The glandular stomachs in our model rats were intact; therefore, the physiological reflux pathway in our rats was similar to that in humans. However, in other models, anastomosis of the glandular stomach and jejunum, similar to Billroth II surgery in humans, was performed; therefore, the intensity of bile reflux may be greater than that of physiological reflux, leading to a high incidence of gastric cancer. Moreover, tumors were clinically found to be prone to develop at the junction of two different types of epithelium. In other models, gastric cancer mainly developed around the mucosa of the gastrojejunal stoma (20-22).

Ulcers occurred spontaneously in the gastric antrum of W/WV mice, and ulcer formation was considered relevant to bile reflux (24, 25). Kaminishi et al. established a rat model of bile reflux, which is similar to ours; in their model, gastric ulcers occurred in all the rats, and the frequency of occurrence increased with time (26). However, in our model, only 1 case of glandular stomach ulceration was observed. This difference in the results may be attributable to the use of different animal models. W/WV mice had defects in the protein tyrosine kinase, thereby resulting in increased sensitivity of the gastric mucosa to stimulation by bile acids. Moreover, Kaminishi et al. used Wistar rats, whereas we used Sprague–Dawley rats.

In our model, only 1 case of intestinal metaplasia of the glandular stomach was found, which is much lower than that of other studies (20-22). So-

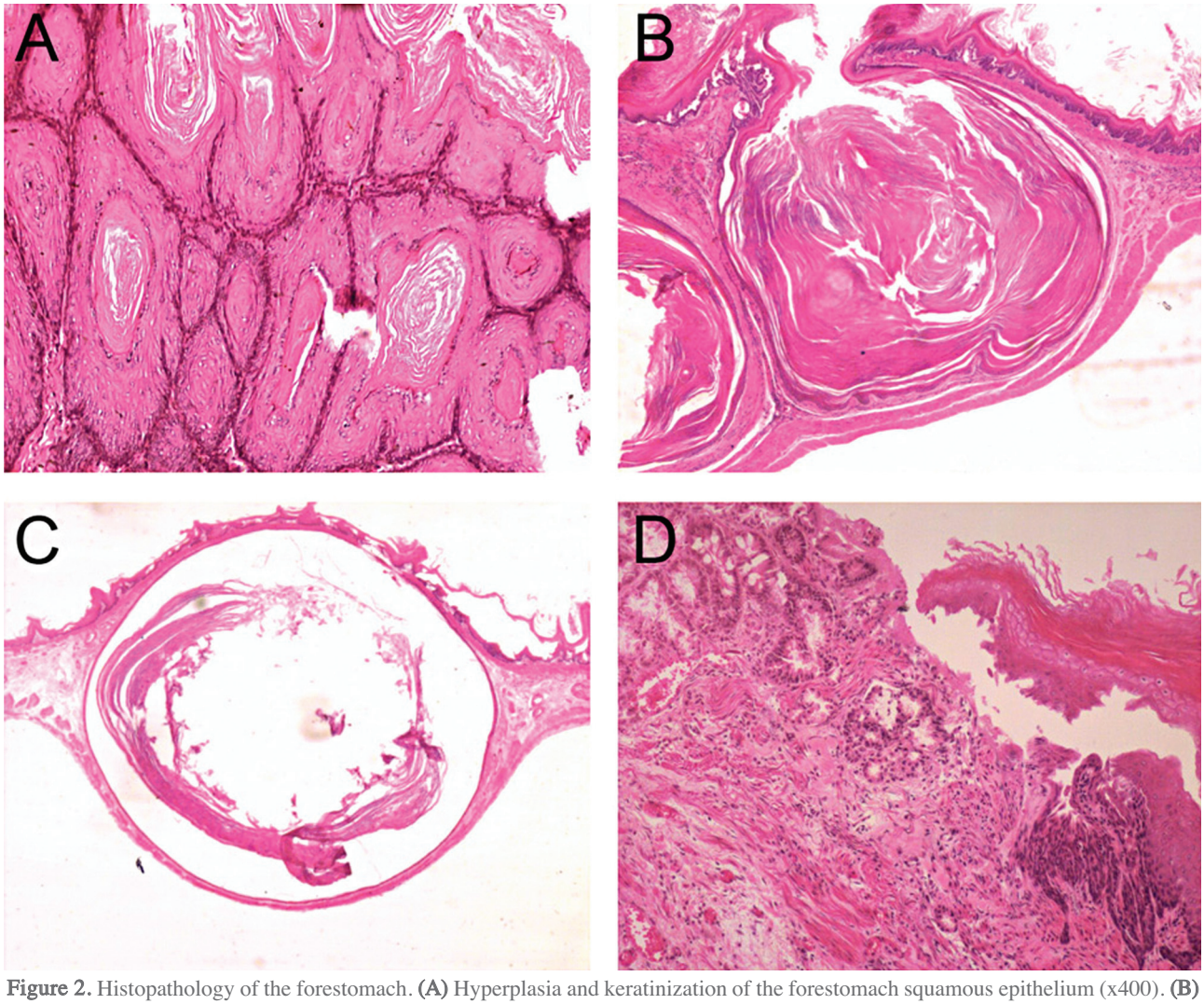

Figure 2. Histopathology of the forestomach. **(A)** Hyperplasia and keratinization of the forestomach squamous epithelium (x400). **(B)** Keratin pearls in the forestomach squamous epithelium (x100). **(C)** Keratocysts in the forestomach (x100). **(D)** Intestinal metaplasia of the forestomach squamous epithelium (x200).

me researchers believe that intestinal metaplasia was an adaptive change of the gastric mucosa. When the local microenvironment of the gastric mucosa was similar to that of the intestine, intestinal metaplasia developed at the gastric mucosa to reduce the damage caused by the alkaline intestinal fluid. When rat stomach wall with vascular pedicle was transplanted to the intestinal wall, typical intestinal metaplasia developed at the transplanted mucosa. The pH of the transplanted mucosa was greater than 6, which is significantly higher than that of the stomach (usually <4.5) (18). The intensity of bile reflux around the gastrojejunal stoma may have been greater in the other models than in our model. This may have resulted in the significant increase in the pH of the local mucosal microenvironment and led to the higher incidence of intestinal metaplasia.

In our model, the squamous epithelium showed significant hyperplasia and papillary bulge, which was also found in the animal models of other studies (27-31). However, to our knowledge, this is the first study to show the formation of keratin pearls and keratocysts caused by epithelial hyperplasia in an animal model for chronic bile reflux.

Other studies have found that chronic bile reflux could cause Barrett's esophagus and esophageal adenocarcinoma (27, 29, 32); however, only 1 case of intestinal metaplasia and no adenocarcinoma occurred for up to 1 year in our rats. This difference in the results may also be attributable to the different operations performed in the studies. In other studies, esophagoduodenal or esophagojejunal anastomosis was performed, leading to higher bile reflux intensity and a favorable environment for the formation of intestinal metaplasia and ade-

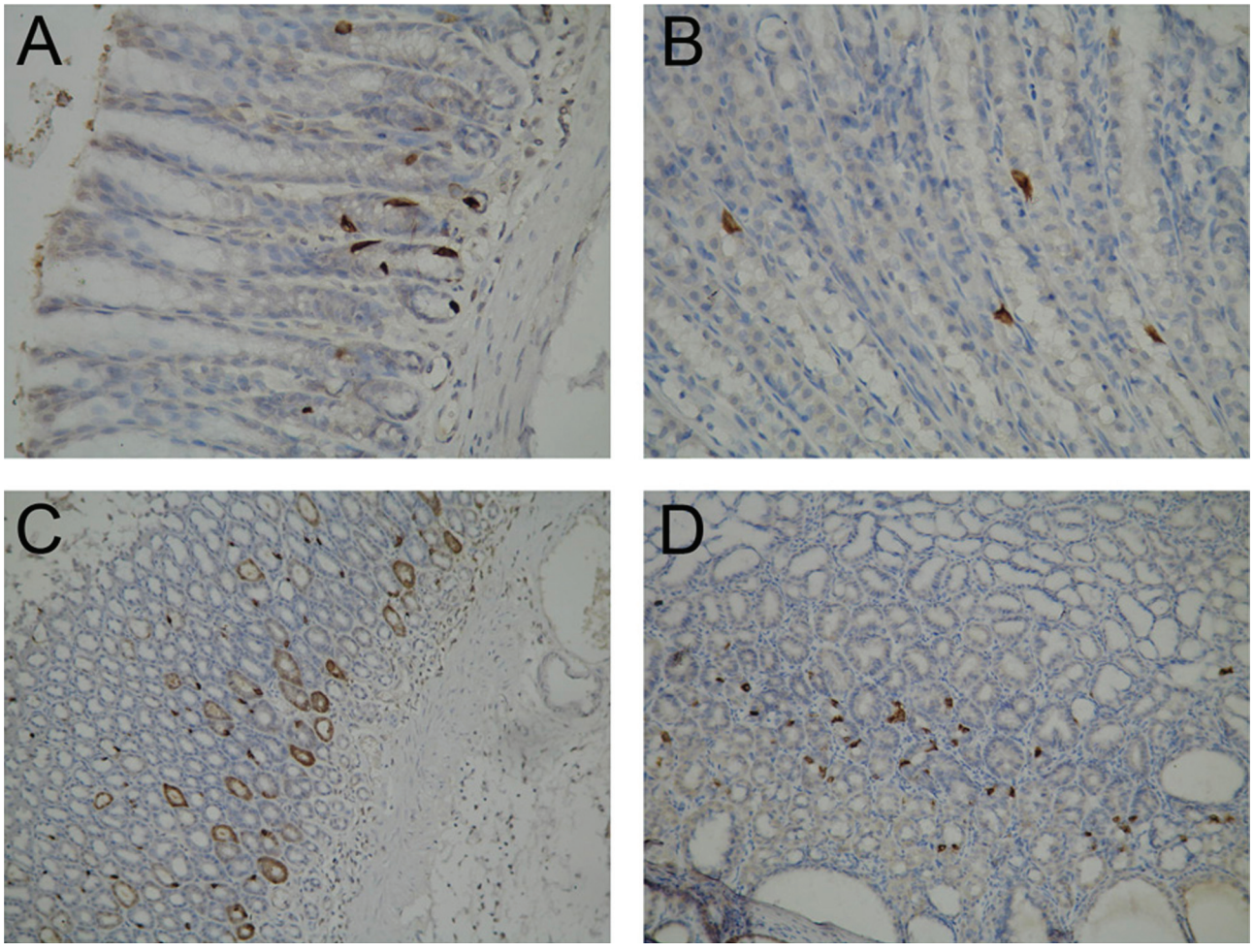

Figure 3. Immunohistochemistry of gastrin. **A** Scattered gastrin-positive cells in the gastric antral epithelium of the control rats (x400). **B** A few scattered gastrin-positive cells in the mucosa of the glandular stomach body of the control rats (x400). **C** Hyperplasia of the gland and the gastrin-positive cells in the gastric antrum of rats who showed chronic bile reflux (x200). **D** Hyperplasia of the gastrin-positive cells in the mucosa of the glandular stomach body along with hyperplasia and expansion of the gland in rats who showed chronic bile reflux (x100).

nocarcinoma. Furthermore, use of omeprazole to block gastric acid secretion was found to increase the incidence of stomach cancer caused by bile reflux (23) and promote the proliferation of the gastric and esophageal epithelium caused by bile reflux (30). This suggests that gastric acid protects the gastric and esophageal epithelium from the damage caused by bile reflux. In our model, bile refluxed through the glandular stomach, which can secrete sufficient gastric acid, and reached the forestomach squamous epithelium; therefore, the toxicity of bile acids can be reduced by gastric acid.

It was also reported that bile reflux could cause hypergastrinemia (26, 30, 33, 34) and inhibit the release of somatostatin (35). Decreased serum levels of somatostatin increased the severity of the symptoms of hypergastrinemia, and in return, hypergastrinemia could increase bile reflux (36). Six months after cholecystectomy, the number of G cells in the gastric antral mucosa increased, combined with significant bile reflux gastritis and foveolar epithelial hyperplasia (37). This suggested that bile reflux can cause G-cell hyperplasia and hypergastrinemia, resulting in epithelial hyperplasia. Using immunohistochemistry, we found that the G cells in both glandular gastric antrum and body showed significant hyperplasia.

The cholecystokinin-2 (CCK-2) receptor is a gastrin-specific receptor and is mainly expressed in the gastric parietal cells and enterochromaffin-like (ECL) cells (38-40). When rats were administered an intravenous injection of gastrin to activate the CCK-2 receptor, heparin-binding epidermal growth factor (HB-EGF) and amphiregulin expression increased, leading to gastric epithelial hyperplasia (41). Goldenring et al. established a rat model for reversible drug-induced oxyntic gland atrophy and found that parietal cells were key to maintaining gastric epithelial homeostasis (42). Beales found that activation of the CCK-2 receptor on the parietal cells by gastrin could induce the secretion of HB-EGF and amphiregulin (43). Therefore, we believe that chronic bile reflux led to G-cell hyperplasia and caused hypergastrinemia. Circulating gastrin activated the CCK-2 receptor on the parietal cells, resulting in the secretion of HB-EGF and amphiregulin; HB-EGF and amphiregulin induced the proliferation of the gastric epithelium and might have led to glandular dysplasia and cancer formation.

Haigh et al. found that the CCK-2 receptor was also expressed in the normal esophagus, reflux esophagitis, Barrett's esophagus, and esophageal adenocarcinoma (44). Therefore, we believe that high levels of circulating gastrin resulting from chronic bile reflux may cause proliferation of the forestomach squamous epithelium through the same mechanism.

However, in one clinical study it was found that in patients with presumed duodenogastric reflux the number of gastrin cells in the gastric antrum was not different from that in controls, or even decreased. A diagnosis of bile reflux gastritis cannot be established only on the basis of several days of bilious emesis and visualized bile in the stomach by endoscopy at one time point. The definition of bile reflux gastritis must exclude any other obvious influencing factors even beyond *H. pylori* infection or medication use, including pathogens, alcohol, eating habits, et cetera.

In summary, we established a rat model simulating the physiological bile reflux pathway shown by humans without foregut surgery. Chronic bile reflux caused significant hyperplasia and expansion of gastric gland in the glandular stomach, and squamous epithelial hyperplasia and keratinization in the forestomach. Epithelial proliferation may be related to G-cell hyperplasia, which was caused by chronic bile reflux. However, the incidences of ulcer, intestinal metaplasia, dysplasia or cancer formation in this model were significantly lower than those reported in the literature.

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Conflict of interest: *The authors declare that they have no conflict of interest.*

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